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Title: On the serological specificity of subunits of two arboviruses of group A (Sindbis and WEE). (Über die serologische Spezifitat von Untereinheiten zweier Arboviren der Gruppe A (Sindbiss und WEE)).

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March 1969

From the results of previous investigators (1,2), it can be concluded that the spherical Sindbis virus possesses a diameter of 60 to 70 mu and consists of a sheath and an inner core. The virus produces along with its infectivity a hemagglutinating and a complement fixing activity. Treatment of the Sindbis virus with Tween 80 and ether results in the lost of infectivity and the liberation of so-called cleavage products; one of these cleavage products is the "hemagglutinin" which is represented as a fractional part of the virus sheath and is the inducer of hemagglutinating and complement-fixing activities. After treatment with cobra snake venom (cobra toxin), the complement fixing units can be isolated. On the basis of flotation density data, these have been regarded as viral inner bodies and have been described as "cores" in the following investigations.

In the present work is studied the serological behavior of four subunits of the Sindbis virus in reactions with antibodies against cleavage products of the Sindbis virus as well as with those of the virus of American equine encephalitis, western type (WEE) which is serologically related to it. In the case of the four subunits, one is the Sindbis virus itself. The others are the "hemagglutinin" and the so-called 1.345 and 1.300"cores". These numbers represent the flotation density of the subunits obtained from CsCl density gradient centrifugation. The serological reactions were determined by neutralization and hemagglutination-inhibition studies as well as by studying complement fixation reactions. In addition, experiments were carried out using the corresponding subunits from the WEE virus. The objective of these investigations was to determine if the four previously mentioned viral subunits differed with regard to their serological specificity. The answer to this question appeared to be of some interest to us in view of the present problems in the preparation of type- and group-specific

antigens of the arboviruses.

MATERIALS AND METHODS

Virus

We employed a Sindbis virus strain (AR 86; 63rd mouse passage) which had been adapted to mouse brain. This strain was passed through cell cultures of chick embryos in our laboratories. In the studies reported on here, we employed materials from the 20th and 25th cell culture passages. The WEE virus originated from the former "Animal Disease and Parasite Research Branch" of the U.S. Department of Agriculture. It had been passed by us four times in fertilized hen eggs and 25 times in cell cultures of chick embryos.

Virus Production, Virus Titration, Hemagglutination-Inhibition Studies and Complement Fixation Reactions

For the production of relatively high titered tissue culture fluids, the preparation of virus concentrates, and for the titration of infectivity using the plaque test, we employed those procedures already described (1). Also the methods used for carrying out the complement fixation reaction (CFR), Hemaggltttination test (HA), and the hemagglutination-inhibition studies (HAI) were drscribed in our previous papers (1,2). We departed from the previously employed CFR procedure in that we not only varied the complement (C') concentration but also either the antigen or the immune serum. Both reagents were diluted two-fold in the CFR buffer given by Mayer et al. (4).

Virus Neutralization Studies

The virus neutralization test. (NT) was carried out using essentially the "checkerboard" method described by Westaway (3). The test was carried out using cell cultures prepared from chicken embryos. Ten-fold dilutions of the virus were prepared in isotonic NaCl - M/90 phosphate buffer, pH 7.2, From each

virus dilution, 0.5 ml aliquots were mixed with an equal volume of a dilution of one of the immune sera (IS, see below) or with normal serum (NS). In this case, we used serum diluted at 2-step intervals going out to a dilution of 1:10 (studies with Sindbis virus) or 1:20 (studies with WEE virus). The virus-serum mixtures were held for one hour at 37°C in a waterbath. From each mixture, two cell culture cups were inoculated with 0.2 ml each. After incubation at 37°C in an incubator for 30 minutes, the cells were washed and then covered with an "overlay" medium which we used in the plaque test (1). The plaques that appeared were enumerated two days later. In the mixture containing normal serum the number of plaques for several dilutions of serum remained within the expected deviation. Thus, it was not dependent on the NS dilution. The average of the plaque counts for a given virus dilution in the presence of different NS concentrations could be used as the reference V_o (virus concentration) for that virus dilution.

Production of Hyperimmune Sera

Sindbis and WEE viruses were grown in primary-cell cultures from hamster kidney. More precise information concerning the production and procedures can be found in one of the previously mentioned works (2). The harvested virus was concentrated by ultracentrifugation and then treated with Tween 80 and ether (1). An aliquot of the products obtained was mixed with an equal volume of Freund's complete adjuvant and used for the immunization of rabbits according to the following immunization schedules:

(a) Sindbis virus. Two ml subcutaneously and then eight days later repitition of this injection; four months after the first injection, 1.5 ml of the Tween-ether treated virus material was injected intravenously and this injection was repeated two months later; six days after the last injection, blood was withdrawn from the ear vein.

(b) WEE Virus. Two ml of a Tween-ether treated virus concentrate mixed with Freund's adjuvant was injected subcutaneously. After 18 days, an intramuscular injection and a subcutaneous injection each containing 2 ml of the same preparation were injected; 14 days later, blood was withdrawn from the rabbit.

The sera obtained in these procedures were inactivated at 56°C for ½ hr and were designated as Sindbis-IS or WEE-IS. A serum obtained from a non-immunized rabbit was used as the normal serum (NS).

Density Gradient Centrifugation and Electron Microscopy

The methods which we used for CsCl density gradient centrifugation and the preparation of fractions have already been described (1,2). The same methods were used for the preparation of negatively stained preparations for electron microscopy.

Production of Virus-specific Subunits

(a)"Complete Virus". The virus concentrates obtained by ultracentrifugation (1) were mixed with CsCl-saturated borate buffer, pH 9.0. The volume ratio was such that the final mixture had a refractive index of $n_d^{20°C} = 1.3575$. We determined the refractive indices with an Abbe refractometer (Zeiss, Oberkochen, BRD). 4.5 ml of the mixture were placed in each of three Lusteroid-cellulose tubes and were centrifuged for 15 hours at 35,000 rpm in a Spinco model L ultracentrifuge employing a SW-39 rotor. From each tube, ten fractions were obtained which had refractive indices between 1.3555 and 1.3585. From our previous studies (1,2), it was known that fractions collected from the mixture in this manner contain "complete" Sindbis virus; therefore, the designation, "complete" virus, will be understood to mean this.

(b)"Hemagglutinin". "Complete" virus was diluted 1:2 with borate buffer, pH 9, treated with Tween 80 and ether (1) and then mixed with CsCl-saturated borate buffer, pH 9.0 until a refractive index of $n_d^{20°C}$ = 1.3600 was achieved. It was then treated as in the preparation of "complete virus". However, now fractions in the $n_d^{20°C}$ range of 1.3585 to 1.3615 were selected; these contain the non-infectious "hemagglutinin" (1).

(c) 1.354- and 1.300- "cores". In these cases, we began with "complete virus" which was mixed with borate buffer, pH 9, containing 5 mg of cobra toxin per ml (Hynson, Westcott, and Dunning, Inc., Baltimore, Md.) in a ratio of 1:2. After incubation with occasional shaking at 37°C for 20 minutes in a water bath, the mixture was diluted with Cs-Cl-saturated borate buffer, pH 9.0, to a $n_d^{20°C}$ value of 1.3640. After 15 hours of centrifugation as previously described for the preparation of "complete virus", we obtained fractions with a $n_d^{20°C}$ value of 1.3650 to 1.380 and 1.3620 to 1.3640. The first fraction contained with 1.345-"cores" and the latter the 1.3000-"cores" (2).

"Complete virus", "hemagglutinin", and 1.345 and 1.300 "cores" were diluted 1:5, 1:10 or 1:20 in the CFR buffer described by Mayer et al. (4) and dialyzed against this buffer.

RESULTS

Studies on The Electron Optical Description of The "Cores"

Photographs prepared electron-optically of "complete Sindbis viruses" and "hemagglutinins" cleaved from them by Tween-ether treatment have already been presented (1). These morphological investigations were supported by the electron optical studies of the virus-specific units designated as "cores". This has already been reported on (2) with photographs but without the designation "core". This happened because the particles that were detected in the fractions with

densities of 1.346 and 1.307 did not shown any special structural characteristics which would allow a conclusion to be drawn with regard to their role in the structure of the virus. This was particularly true of the particles in the 1.346-fraction which had diameters of 2 to 8 mu. We obtained somewhat better photographs of the particles which were present in the 1.307-fraction. These are shown in Fig. 1 and can be observed to have a round shape and diameters from 6 to 20 mμ. The magnitude of these diameters indicates, if one keeps in mind the structure of the "complete" Sindbis virus (1), that one is not dealing with intact inner bodies.

Fig. 1. Particles in the 1.307-fraction obtained by CsCl density gradient centrifugation after treatment of "complete" Sindbis virus with cobra toxin. Negative contrast staining with phosphotungestic acid. 100,000 X magnification. (The reader is referred to the original journal for a picture of the particles).

The Serological Specificity of the Four Subunits

(a) Neutralization Studies

Firstly, the reaction between "complete" Sindbis virus and Sindbis-IS and WEE-IS were examined using the neutralization test. Fig. 2 shows the results obtained in these studies. The graph shows that a linear relationship is obtained in the area of the IS dilution tested when one plots the log of the decrease in infectivity titer against the log of the hyperimmune serum concentration. In contrast, only a very small fraction of the Sindbis virus was neutralised by the WEE-IS.

Quite a different situation with regards to serological specificity was noted in the case of the neutralization of "complete" WEE-virus by homologous WEE-IS and heterologous Sindbis-IS. As shown in Fig. 3, both sera were equally effective in the neutralization of WEE virus. Also, here, the linear relationship

previously mentioned was found between the log of the infectivity titer decrease and the log of the IS concentration. In addition, it was noted that the slopes of the lines of the neutralization curves obtained with Sindbis virus/Sindbis IS, WEE virus/ WEE-IS, and WEE virus/Sindbis-IS were the same.

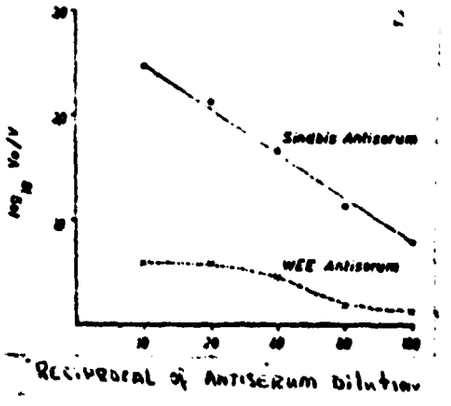

Fig. 2. Neutralization studies with "complete" Sindbis virus and Sindbis immune serum and WEE immune serum. Log_{10} (V_o/V) is the ratio of the plaque count with normal serum to the plaque count with immune serum.

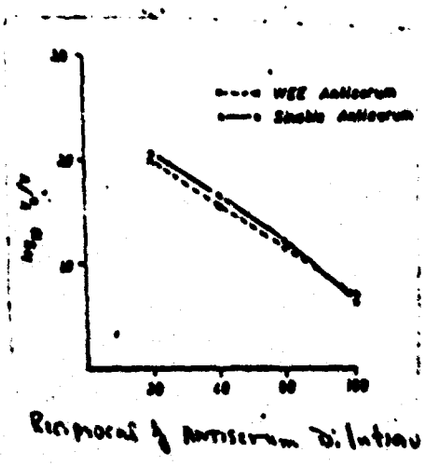

Fig. 3. Neutralization studies with "complete" WEE virus and WEE immune serum and Sindbis immune serum. See legend for Fig. 2.

(b) Hemagglutination-Inhibition Studies

Two of the four viral subunits that were prepared showed hemagglutinating activity: the "complete" virus particle and the "hemagglutinin". Both components of the Sindbis virus and the WEE virus were studied using Sindbis-IS and WEE-IS in the hemagglutination-inhibition reaction. The results that were obtained from these studies are shown in Table I. It is evident that "complete" Sindbis virus reacts four times more strongly with homologous hyperimmune serum than with heterologous hyperimmune serum (first vertical column). On the other hand, the hemagglutinating activity was inhibited to the same degree by both WEE-IS and Sindbis-IS (second vertical column). Similar results were obtained also for the Hemagglutinins isolated from the Sindbis and WEE viruses (third and fourth vertical columns).

TABLE I

Results of the cross-hemagglutination-inhibition tests with "complete" viruses and "hemagglutinins" of Sindbis and WEE viruses.

Hemagglutinating antigen	"Complete" virus		"Hemagglutinin"	
Antiserum	Sindbis	WEE	Sindbis	WEE
Sindbis-IS	320*	320	1280	320
WEE-IS	80	320	320	320

* Reciprocal of the inhibition titer.

(c) Complement Fixation Reactions

The serological specificity of all four subunits isolated from the Sindbis and WEE viruses was determined ultimately using the complement fixation reaction. The curves on the left side of Fig. 4 illustrate the results that were obtained with the Sindbis virus subunits when the reactions were carried out using various IS dilutions and a constant antigen dilution.

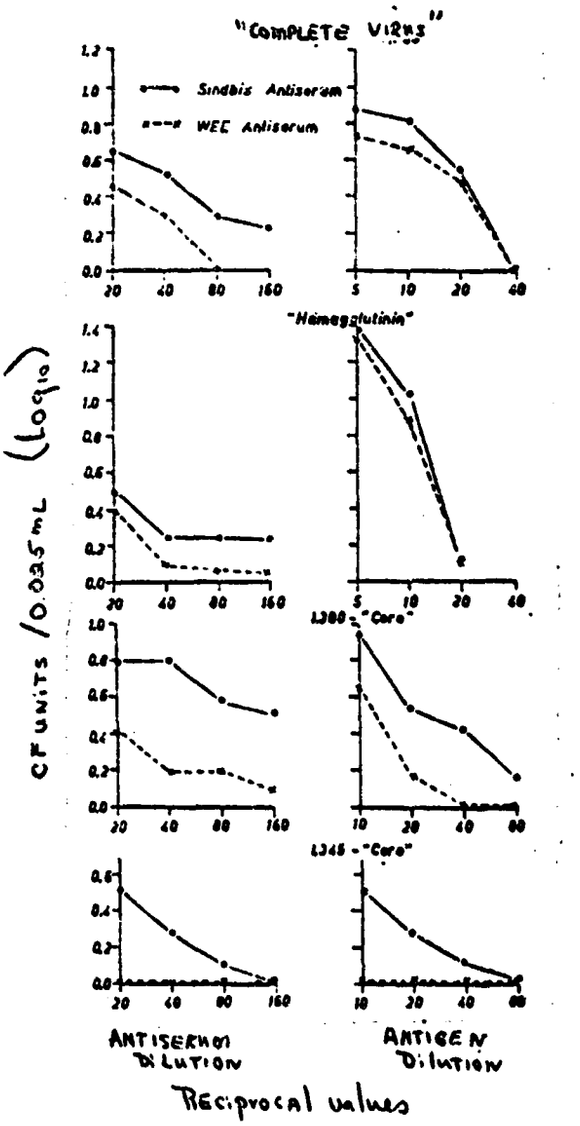

Fig. 4. Complement fixation reactions with four subunits of Sindbis virus with Sindbis immune serum and WEE immune serum. The concentration of antiserum (left side) and the antigen concentration (right side) were varied.

The curves in Fig. 4, particularly those on the right side, demonstrate that "complete" Sindbis virus and Sindbis "hemagglutinin" react with Sindbis IS as well as with WEE-IS in the complement fixation reaction. These cross-reactions were not as strongly expressed when the "1.300-cores" were used as the testing antigen. The "1.345-cores" fixed C' only in the presence of homologous antiserum but not in the presence of heterologous antiserum (WEE-IS). Fig. 5. shows the corresponding results obtained with viral subunits from WEE. In the studies with WEE virus, we employed only increasing dilutions of the individual antigen components and a constant IS concentration since the cross-reactions could be more easily detected in this manner than when the combination of differing IS-dilutions and a constant antigen concentration was employed. This fact is shown in Fig. 4. for the experiments carried out using the Sindbis subunits. From Fig. 5, it was concluded that, in contrast to the Sindbis virus system, not only in two, but in all of the four WEE virus subunits tested, a very distinct cross-reaction with heterologous IS was noted.

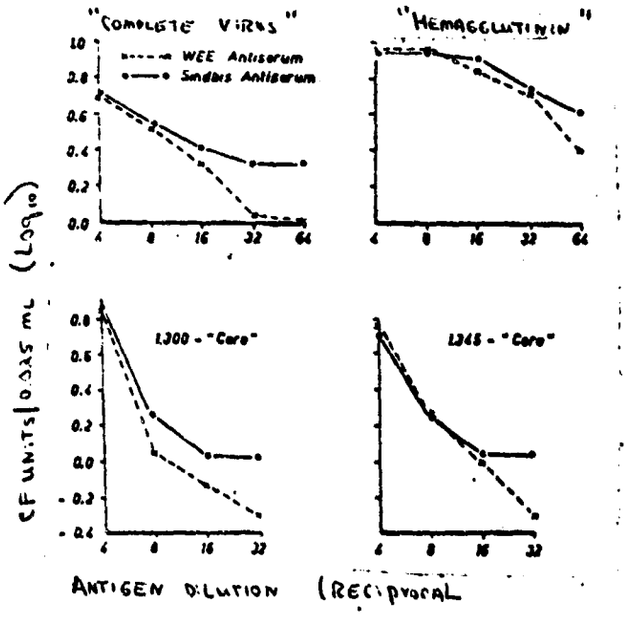

Fig. 5. Complement fixation reactions of the four subunits of WEE virus. The dilutions of WEE and Sindbis immune sera were ~~chan~~ and the dilution of antigen was changed.

DISCUSSION

From the results that have been presented, it is obvious that IS prepared against the cleavage products of the Sindbis virus will neutralize WEE virus particles as well as it does the virus particles of the Sindbis virus, whereas a corresponding WEE-IS neutralizes the homologous WEE virus but not the Sindbis virus to the same degree observed in the first system. A so-called antigenic uni-directional relationship is proposed as was already postulated (5) on the basis of studies using the plaque reduction test in the Sindbis-WEE system. This uni-directional relationship was expressed only weakly in the examination of the hemagglutinating subunits of the Sindbis and WEE viruses during the hemagglutination-inhibition studies; in contrast, a reciprocal relationship between the viruses was demonstrated in this case. A close reciprocal relationship between "complete" Sindbis and WEE virus particles and their respective "hemagglutinins" was also observed in the complement fixation reaction. In contrast, using this reaction, the existence of a uni-directional relationship could be detected, in the direction indicated earlier, between the 1.300- and 1.345- "cores", particularly with the latter.

These results provide evidence as to the antigenic composition of the viral subunits that have been tested. It has been assumed that the majority of the groups present on the surfaces of the Sindbis and WEE virus particles, which possess hemagglutinating and complement-fixing activity, are the same or are very similar with regard to their antigenic structure. In addition, however, there appears that on the surfaces of the particles of both virus species, there are structures which demonstrate observable differences and exhibit a so-called uni-directional relationship as shown during the neutralization studies. A relationship of this kind could be theoretically explained by the acceptance of "sterically hindered determinants" (6). It is possible that the Sindbis virus possesses on its surface structures A and b which are active and necessary for infectivity, whereas in the case of the WEE virus, the determinants B and a

are the antigens responsible for this function. The small letter should stand for sterically hindered determinants which are immunologically inactive but are still capable of reacting specially with antibodies. Along with this assumption, it can be concluded that the Sindbis virus stimulates the formation of A antibodies which are capable of reacting both with the A determinant of Sindbis virus and with the a determinant of WEE resulting in the neutralisation of both virus types. Conversely, the WEE virus induces the production of C antibodies which react only with the C determinants of the WEE virus; no reaction is observed with the Sindbis virus as it does not possess the C or c determinants. Consequently, the Sindbis virus is not neutralized by WEE-IS which neutralizes only the WEE virus. In an analogous manner, the uni-directional relationshp of the cores, as detected through the use of complement fixation reactions, could be represented. Another explanation of the uni-directional relationship can be found in the concept that an antigenic determinant on the Sindbis virus induces the formation of antibodies which react with this determinant and with a "related determinant" which is found on the WEE virus and is antigenically related to the determinant of the Sindbis virus. Inversely, however, antibodies formed against the "related determinant" of the WEE virus react only with it and not with the determinant found on the Sindbis virus. This concept is based on the three-dimensional model of the antibody molecule proposed by Schulze (7) to explain the activity of heterologous antibodies. In addition, it was considered more an exception than an explanation that Sindbis antibodies and WEE antibodies react with particles of both virus species and that in the third component of the neutralization reaction, namely in the cultured cells, the WEE virus particles charged with Sindbis antibodies as well as with WEE antibodies are not infectious, whereas the complex, WEE antibodies/Sindbis virus particles, dissolves in the cells and leads to infection by the Sindbis virus. This mode of thought

is objectionable, however, since the uni-directional relationship is detected not only in the neutralization studies but also in the complement fixation reaction with 1.345-"cores".

Finally, in the interpretation of the uni-directional relationship, one must consider the possibility that during immunization, Sindbis-IS was produced which had antibodies with a broader specificity than those in the WEE-IS. This, however, would be objectionable in view of the results of the hemagglutination-Inhibition studies and complement fixation reactions with "complete" viruses and "hemagglutinins". In these cases, distinct cross-reactions were observed. Moreover, the identical slopes of the various neutralization curves indicate that the heterologous Sindbis antibodies react as well with the WEE viral particles as does the homologous WEE antibodies (8).

SUMMARY

The serological specificity of four subunits of Sindbis and WEE virus was investigated in cross-reactions. The four subunits were: "complete" virus particles, the "hemagglutinin", and the so-called 1.300- and 1.345-"cores". In neutralization studies, a uni-directional relationship between Sindbis and WEE virus was demonstrated, in which Sindbis antibodies neutralized Sindbis and WEE virus, whereas WEE antibodies reacted with the homologous virus only. An analogous relationship was also found in the CF reaction with 1.300- and 1.345 "cores", especially with the latter. On the other hand, "complete" virus particles and "hemagglutinin" showed clear-cut cross-reactions when used in CF reactions.

The results are discussed in relation to the antigenic structure of the two viruses and to the uni-directional relationship which has been demonstrated for certain subunits.

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